Cell, Vol. 99, 35–46, October 1, 1999, Copyright ©1999 by Cell Press

Cosuppression of Nonhomologous Transgenes in *Drosophila* Involves Mutually Related Endogenous Sequences

Manika Pal-Bhadra,† Utpal Bhadra,† and James A. Birchler*
Division of Biological Sciences
University of Missouri
Columbia, Missouri 65211

Summary

Cosuppression refers to the phenomenon in which silencing among dispersed homologous genes occurs. Here we demonstrate that two nonhomologous reciprocal fusion genes, *white-Alcohol dehydrogenase* (*w-Adh*) and *Adh-w*, exhibit cosuppression using the endogenous *Adh* sequence as an intermediary. Deletion of the endogenous *Adh* gene eliminates the interaction, while reintroduction of an 8.6 kb *Adh* fragment restores the silencing. Using truncated *Adh* constructs, a nontranscribed segment in the *Adh* regulatory region was found to be one of the sequences required for homology recognition. The silencing interaction is initiated during early development. The silenced transgenes are associated with the Polycomb group complex of chromatin proteins.

Introduction

There is increasing evidence from the study of ectopic transgenes that mechanisms exist in higher eukaryotic organisms to inactivate multiple copies of genes that produce an overexpression or abnormal transcription (Matzke et al., 1989; Napoli et al., 1990; van der Krol et al., 1990). These systems may have evolved as a means to prevent high levels of expression of transposable elements, and hence high mutation rates (Flavell, 1994; Chaboissier et al., 1998; Jensen et al., 1999), or to inhibit the expression of viruses (Jorgensen et al., 1998; Voinnet et al., 1998). In both fungi and plants, there is evidence that mechanisms involving either DNA–DNA association (Meyer et al., 1993; Matzke and Matzke, 1995; Selker, 1999) or posttranscriptional RNA turnover (Cogoni and Macino, 1997; Vaucheret et al., 1998) can be involved.

In *Ascobolus*, methylation-associated silencing can be transferred between a silenced and a naive allele during meiosis in a manner reminiscent of gene conversion, which implies a contact between alleles for the transfer of the silencing signal (Colot et al., 1996). On the other hand, in *Neurospora crassa* the phenomenon of quelling, in which a transgene in only one nucleus of a dikaryon can render all homologous copies silenced, indicates a mechanism involving RNA metabolism rather than homologous gene association (Cogoni et al., 1996). Also, in plants there is evidence for transcriptional (Matzke and Matzke, 1995) and posttranscriptional (Baulcombe and English, 1996) processes involved with transgene silencing. Although under some circumstances both types of mechanisms may potentially be intertwined in a particular case, the aforementioned studies in fungi illustrate that they can be distinguished.

Recently, transgene silencing and related effects (Pal Bhadra et al., 1997; Chaboissier et al., 1998; Fire et al., 1998; Kennerdell and Carthew, 1998; Ruiz et al., 1998; Bahramian and Zarbl, 1999; Jensen et al., 1999) have been described for animal species. Here also is evidence for transcriptional and posttranscriptional mechanisms. The case of *white-Alcohol dehydrogenase* (*w-Adh*) transgenes in *Drosophila melanogaster* involves an accumulation of the repressive Polycomb group (PcG) chromatin complex on the silenced copies, implying a transcriptional mechanism (Pal Bhadra et al., 1997). In contrast, RNA interference involving double-stranded RNA is most readily interpreted as a posttranscriptional process (Fire et al., 1998; Kennerdell and Carthew, 1998; Montgomery et al., 1998).

In this article, we report that insertion of multiple *w-Adh* copies into different locations in the *Drosophila* genome strongly suppresses the activity of the reciprocal *Adh-w* construct by triggering the accumulation of the PcG complex on the transgenes. We refer to this unique interaction as "nonhomologous cosuppression," following the definition of cosuppression by Jorgensen (1995) as when "dispersed homologous genes are suppressed in their expression." The silencing signal between the nonhomologous sequences occurs through an intermediary endogenous gene that is homologous to portions of both transgenes.

The endogenous sequences that mediate the interaction involve at least one site in the transcribed portion of *Adh* and another in the nontranscribed regulatory region. Dissection of the latter reveals that a short segment surrounding the distal enhancer of *Adh* is required for silencing. These findings, together with the fact that the endogenous gene is not expressed in at least some tissues in which *w-Adh* and *Adh-w* are silenced, argue for a transcriptional suppression based on homology recognition. The evidence indicates a process distinct from one involving RNA-mediated silencing, such as occurs with RNA interference, and indicates the presence of multiple mechanisms of dispersed gene silencing in the animal kingdom.

Results

Multiple *w-Adh* Copies Eliminate *Adh-w* Expression

Genomic sequences of the *white* eye color gene and the *Adh* gene were separated into two fragments and rejoined to produce reciprocal constructs as described previously (Birchler et al., 1990; Rabinow et al., 1991; Pal Bhadra et al., 1997). The 2.5 kb *white* regulatory sequence was rejoined with 1.9 kb of the *Adh* structural gene in the *w-Adh* construct, while a 6.5 kb *white* structural sequence was fused with the 1.8 kb *Adh* promoter fragment in the *Adh-w* construct. The reciprocal transgenes *w-Adh* and *Adh-w* share the same restriction

*To whom correspondence should be addressed (e-mail: birchlerj@missouri.edu).
† These authors contributed equally to this work.

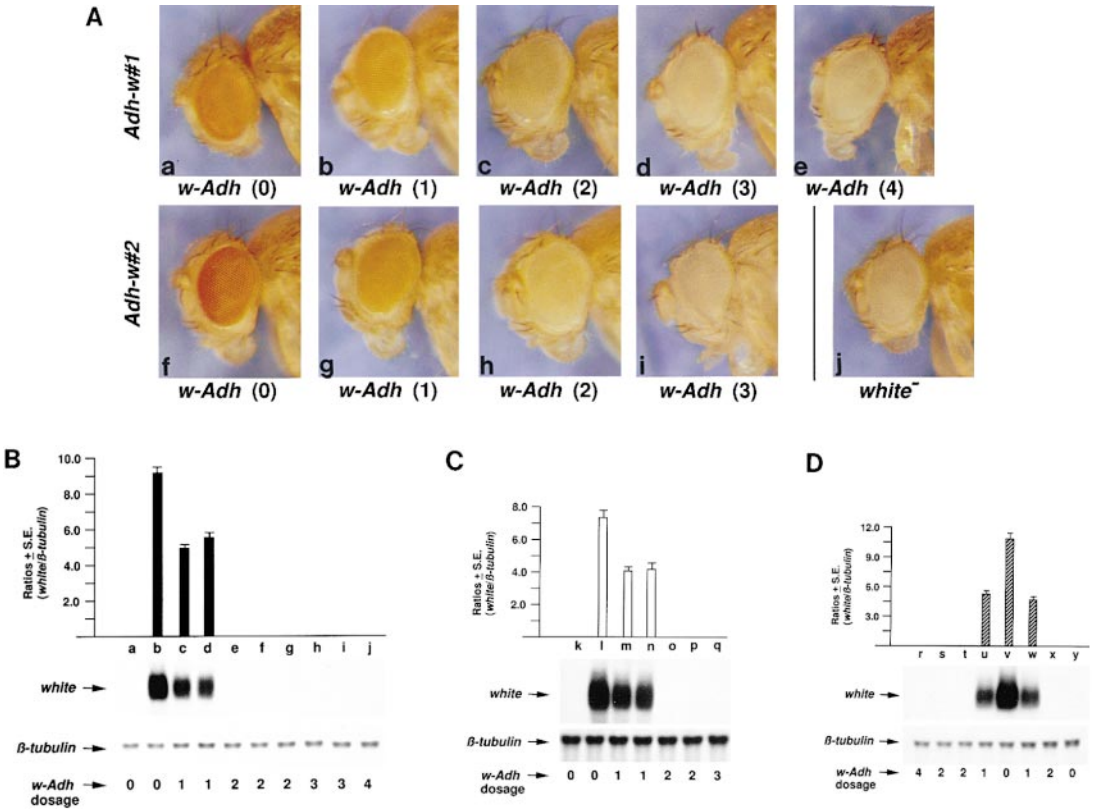


Figure 1. Silencing of *Adh-w* Transgenes with Progressive Increase of *w-Adh* Copies

The cytological locations of the three *w-Adh* transgenes, #1, #2, and #3, are in the 5A, 52A, and 85A regions, respectively, and that of *Adh-w#1* and *#2* are 16B and 70C.

(A) The eye color of *Adh-w* flies is reduced with increasing *w-Adh* dosage. All flies are in a *y* w^{67c23}; Adh^{fn6} background. The *w-Adh* copy number is noted in parentheses.

(B) Autoradiogram of a Northern blot of RNA from *Adh-w#1*/Y adult flies carrying zero to four copies of *w-Adh* transgenes hybridized with antisense *white* RNA. Lanes are (a) *y* w^{67c23}/Y, (b) *Adh-w#1*/Y, (c) *Adh-w#1*/Y; *w-Adh#3*/+, (d) *Adh-w#1*/Y; *w-Adh#2*/+, (e) *Adh-w#1*/Y; *w-Adh#3*/*w-Adh#3*, (f) *Adh-w#1*/Y; *w-Adh#2*/*w-Adh#2*, (g) *Adh-w#1*/Y; *w-Adh#2*/+; *w-Adh#3*/+, (h) *Adh-w#1*/Y; *w-Adh#2*/+; *w-Adh#3*/*w-Adh#3*, (i) *Adh-w#1*/Y; *w-Adh#2*/*w-Adh#2*; *w-Adh#3*/+, and (j) *Adh-w#1*/Y; *w-Adh#2*/*w-Adh#2*; *w-Adh#3*/*w-Adh#3*. The bottom panel shows the reprobing with β-*tubulin* as a gel-loading control. The abundance of *white* RNA of the *Adh-w#1* construct relative to β-*tubulin* is depicted by the bar diagrams, which represent the mean of triplicate blots. Error bars delimit the 95% confidence intervals. Males and females with the same *Adh-w* and *w-Adh* copy number are equally affected. All flies are in a *y* w^{67c23}; Adh^{fn6} background.

(C) An autoradiogram from the Northern analysis using *Adh-w#2*/+ adult flies. Lanes are (k) *y* w^{67c23}/Y, (l)w^{-}/Y; *Adh-w#2*/+, (m)w^{-}/Y; *Adh-w#2*/*w-Adh#3*, (n) w^{-}/Y; *w-Adh#2*/+; *Adh-w#2*/+, (o) w^{-}/Y; *w-Adh#2*/+; *Adh-w#2*/*w-Adh#3*, (p) w^{-}/Y; *w-Adh#2*/*w-Adh#2*; *Adh-w#2*/+, and (q) w^{-}/Y; *w-Adh#2*/*w-Adh#2*; *Adh-w#2*/*w-Adh#3*.

(D) A representative Northern blot using *Adh-w#1* larvae. Lanes are (r) w^{-}*Adh-w#1*/Y; *w-Adh#2*/*w-Adh#2*; *w-Adh#3*/*w-Adh#3*, (s) w^{-} *Adh-w#1*/Y; *w-Adh#3*/*w-Adh#3*, (t) w^{-} *Adh-w#1*/Y; *w-Adh#2*/*w-Adh#2*, (u) w^{-} *Adh-w#1*/Y; *w-Adh#3*/+, (v) w^{-} *Adh-w#1*/Y, (w) w^{-} *Adh-w#1*/Y; *w-Adh#2*/+, (x) w^{-} *Adh-w#1*/Y; *w-Adh#2*/+; *w-Adh#3*/+, and (y) *y* w^{67c23}/Y.

sites from each parental gene at the junction sites; therefore, no sequence is common to both. We used the multiple *w-Adh* transgenic stocks described earlier (Pal Bhadra et al., 1997). The *Adh-w* hybrid construct is present in a *y* w^{67c23} (*white* deletion) background (Birchler et al., 1990) and thus is the source of the eye pigmentation and *white* transcripts in these strains. The initial P element construct was genetically mobilized from the third chromosome to the X using the transposase source in the *delta 2-3* strain (Robertson et al., 1988). As expected, both transformant lines extensively overexpressed *white* transcripts relative to the endogenous *white* gene because *Adh* is more strongly transcribed than *w*. The single inserts (*Adh-w#1* or *Adh-w#2*) are expressed in a w^{67c23}; Adh^{fn6} null background but differ slightly due to positional variation (Figure $1A_{a,f}$). We increased the number of the reciprocal *w-Adh* construct in a background with one copy of *Adh-w*. Addition of a single *w-Adh* transgene in two different locations (52A and 85D) reduces eye color (Figure $1A_{b,g}$) and *white* transcripts to approximately 50% of normal levels (Figures $1B_{c,d}$ and $1C_{m,n}$). Further increase of *w-Adh* copies in this genotype nearly eliminates *white* phenotypic expression in the eye (Figure $1A_{c,h}$) as well as RNA. The results reveal that two *w-Adh* copies in the same or different locations exhibit a similar effect (Figures $1B_{e-g}$ and $1C_{o,p}$). Three or four *w-Adh* copies (the highest number tested) eliminate detectable *white* expression (Figures $1A_{d,e,i}$, $1B_{h-j}$, and $1C_{q}$.). This strong reduction of *Adh-w* expression is more effective than the multiple *w-Adh* interaction reported previously (Pal Bhadra et al., 1997). To determine whether nonhomologous cosuppression is effective in the larval

stage, the level of the *white* transcripts was compared in larvae and adults of the same genotypes (Figure 1D). This Northern profile comparison indicates that silencing is similar in the two developmental stages.

Level of *Adh* mRNA and Nonhomologous Cosuppression

All previously reported cases of silencing of transgenes require homology among the genes involved, making the *w-Adh/Adh-w* interaction unusual. It was suspected that the two transgenes might interact via the endogenous *Adh* gene, which is homologous to portions of both. Multiple *w-Adh* insertions suppress endogenous *Adh* transcripts but have no influence on endogenous *white* (Pal Bhadra et al., 1997). The original phenotypic analysis of *Adh-w* was performed with a wild-type endogenous *Adh* gene heterozygous with the mutant Adh^{fn6} allele present in the *w-Adh*-containing stocks. To determine whether a high level of *Adh* transcripts is required for nonhomologous cosuppression, we replaced the normal *Adh* with the Adh^{fn6} mutant allele, which has a defect at the splicing site in the first intron resulting in an 85%–90% reduction of *Adh* transcripts (Benyajati et al., 1982). We found that the presence of *w-Adh* copies equally cosuppressed *Adh-w* expression both in eye phenotype and in transcript levels in a homozygous Adh^{fn6} background relative to normal (Figure 1). Therefore, a low level of steady-state *Adh* RNA does not affect nonhomologous cosuppression.

Endogenous *Adh* and Nonhomologous Cosuppression

To define the role of endogenous *Adh*, we next deleted the entire *Adh* sequence using two overlapping viable deficiencies, $Df(2L)Adh^{379}$ and $Df(2L)Adh^{fn3}$ (Gubb et al., 1997; J. Roote, personal communication), in flies with either *Adh-w#1* or *Adh-w#2* as the phenotypic reporter (Figure 2A). As a control, the relative abundance of the endogenous *white* transcripts was examined in flies that carry either an *Adh* gene or the Adh^{-} deletions and was found to be nearly equal (Figures $2B_{b,c}$ and $2C_{k,m}$). In addition, deletion of the *Adh* sequences has no effect on the *Adh-w* expression alone (Figure $2B_g$). However, when two copies of *w-Adh* are present in flies with a deleted endogenous *Adh* (*y* w^{67c23} *Adh-w#1*/Y; $Df(2L)Adh^{379}/Df(2L)Adh^{fn3}$; *w-Adh#3/w-Adh#3*), the eye color of these flies is restored to the *Adh-w#1*/Y level (Figure $2A_a$). A similar experiment with the *Adh-w#2* insert using one copy of *w-Adh* (*y* w^{-}/Y; $Df(2L)Adh^{379}/Df(2L)Adh^{fn3}$; *Adh-w#2/w-Adh#3*) showed the same response (Figure $2A_b$). The reduced RNA expression of both *Adh-w* inserts in the presence of multiple *w-Adh* copies is eliminated in *Adh*-deficient flies (Figures $2B_f$ and $2C_p$). Thus, deletion of endogenous *Adh* disrupts the interaction between *w-Adh* and *Adh-w* (Figure 2D).

To test whether the endogenous *Adh* genomic region itself is required for nonhomologous cosuppression, we replaced it with an Adh^{+} transformant containing an 8.6 kb *Adh* fragment (Laurie-Ahlberg and Stam, 1987) inserted at the 67C or 92B regions in an otherwise *Adh-w*/+; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$; *w-Adh*/+ genotype. Analysis of adult flies carrying zero or one copy of the *w-Adh* transgene showed that addition of a full-length Adh^{+} in either location restores the *w-Adh* and *Adh-w* interaction. These results indicate that an 8.6 kb *Adh* fragment is sufficient to mediate cosuppression between *w-Adh* and *Adh-w*.

Endogenous *white* and Nonhomologous Cosuppression

To test whether endogenous *white* could also act as an intermediary, we introduced the w^{1} mutation into the $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$; *Adh-w#2/w-Adh#3* stock and examined the eye color of reporter *Adh-w*/+ flies. The w^{1} mutation retains *white* sequences, but the transcripts are not functional (Zachar and Bingham, 1982). Thus, *Adh-w* is responsible for the eye pigment. The eye color of the above genotype with w^{1} does not differ from that with a *w* deficiency (data not shown). This result demonstrates that *white* structural sequences cannot act as an intermediary for nonhomologous cosuppression.

Role of Endogenous *Adh* as a Homologous Intermediary

w-Adh Cosuppression Is Independent of Endogenous Adh

The expression of *Adh-w* is reduced when *w-Adh* transgenes are added to the genome. To examine whether cosuppression of the *w-Adh* transgenes alone is dependent on the endogenous *Adh* sequences, we quantitated the *w-Adh* transcripts from a two-copy *w-Adh* stock in an Adh^{+} and Adh^{-} background using Northern blot hybridization. In normal *Adh* stocks, one copy of *w-Adh#3* is readily expressed, while two copies of the same construct are reduced in expression (Pal Bhadra et al., 1997). Using the overlapping deficiencies as described above, we deleted the entire *Adh* sequences, but this does not alter the *w-Adh* transcript levels (data not shown), indicating that cosuppression of the *w-Adh* transgenes is independent of endogenous *Adh*. Also, the Polycomb binding of the silenced *w-Adh#3* insert (Pal Bhadra et al., 1997) is not eliminated by deleting endogenous *Adh* (not shown).

A Direct Interaction between a Full-Length Adh Transgene and Adh-w

To define the role of *Adh* as an intermediary, we replaced *w-Adh* with full-length *Adh* transgenes in the same *Adh-w*/+ genotype. The full-length *Adh* directly shares common sequences with *Adh-w* in the regulatory region. The expression of the *Adh-w* reporter is reduced to the same degree in the presence of an *Adh* transgene as was found with *w-Adh* (Figures 3A and 3B).

We next examined the level of *Adh-w* transcripts from flies with deletions of the entire endogenous *Adh* sequences using the same overlapping deficiencies as described above. Interestingly, deletion of the endogenous sequence does not reactivate *Adh-w* expression (Figures 3A and 3B). Therefore, full-length *Adh* transgenes are able to affect the *Adh-w* gene directly. This result suggests that homology between the two types of transgenes in the *Adh* regulatory region is sufficient to trigger silencing.

Minimum Sequence for Homology Recognition

A series of progressively truncated constructs (Corbin and Maniatis, 1989a, 1989b) was tested to identify the

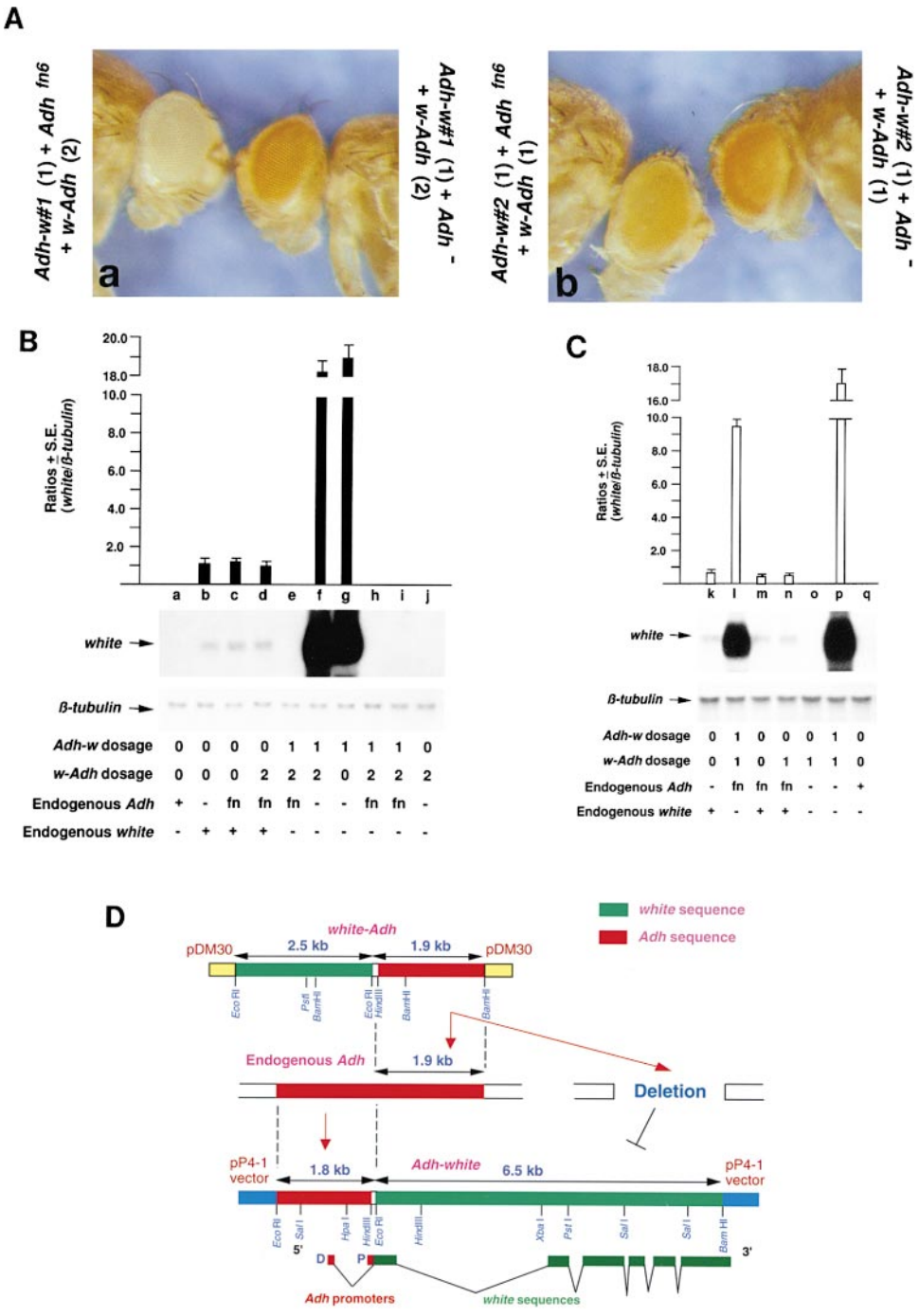


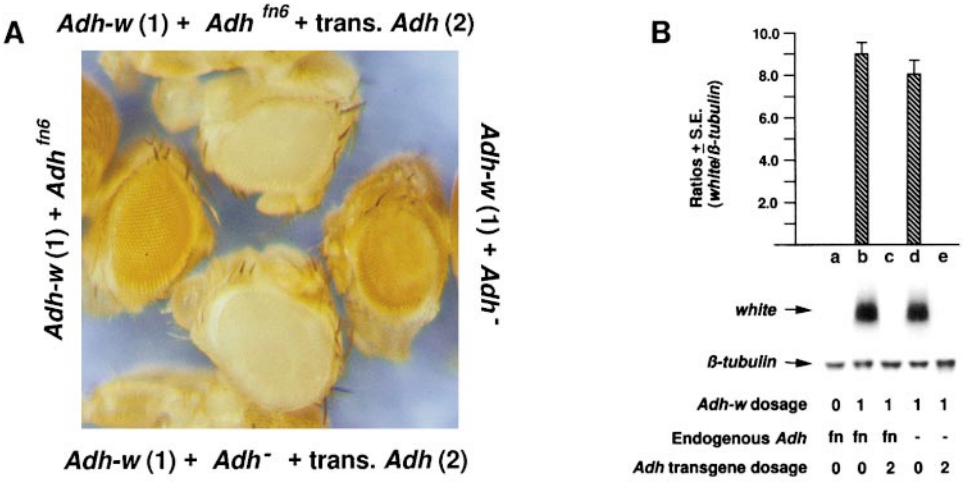
Figure 2. Restoration of Normal *Adh-w* Expression in *Adh*-Deleted Flies in the Presence of *w-Adh* Transgenes

(A) The eye color of *Adh-w* flies, which is reduced in the presence of *w-Adh* inserts, is restored by deletion of the endogenous *Adh* sequences. The copy number of each transgene is noted in parentheses.

(B) Autoradiogram of a Northern blot illustrating the restoration of normal transcript levels of the *Adh-w#1* insert by deletion of the endogenous *Adh* gene. Lanes are (a) $y\ w^{67c23}$/Y, (b) w^{+}/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$, (c) w^{+}/Y; Adh^{fn6}/Adh^{fn6}, (d) w^{+}/Y; *w-Adh#3*/*w-Adh#3*, (e) $y\ w^{67c23}$ *Adh-w#1*/Y; *w-Adh#3*/*w-Adh#3*, (f) $y\ w^{67c23}$ *Adh-w#1*/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$; *w-Adh#3*/*w-Adh#3*, (g) $y\ w^{67c23}$ *Adh-w#1*/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$, (h) $y\ w^{67c23}$ *Adh-w#1*/Y; *w-Adh#2*/*w-Adh#2*, (i) $y\ w^{67c23}$ *Adh-w#1*/Y; *w-Adh#2*/Adh^{fn6}; *w-Adh#3*/+, and (j) $y\ w^{67c23}$/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$; *w-Adh#3*/*w-Adh#3*. All flies except the *Adh* deletion are in an Adh^{fn6} background.

(C) A Northern analysis involving *Adh-w#2* constructs. The lanes are (k) w^{+}^/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$, (l) $y\ w^{67c23}$/Y; *Adh-w#2*/*w-Adh#3*, (m) w^{+}/Y; Adh^{fn6}/Adh^{fn6}, (n) w^{+}/Y; *w-Adh#3*/+, (o) $y\ w^{67c23}$/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$; *w-Adh#3*/+, (p) $y\ w^{67c23}$/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$; *Adh-w#2*/*w-Adh#3*, and (q) $y\ w^{67c23}$/Y.

(D) Regions of sequence homology among the transgenes and endogenous *Adh*. The schematic diagram shows that 1.9 kb of sequence is in common between the *white* promoter *Adh* reporter and the endogenous *Adh*. The *Adh* and *Adh-w* constructs share a 1.8 kb promoter sequence. Deletion of the endogenous *Adh* sequence eliminates the interaction between *w-Adh* and *Adh-w*. D and P, distal and proximal promoters of *Adh*, respectively.



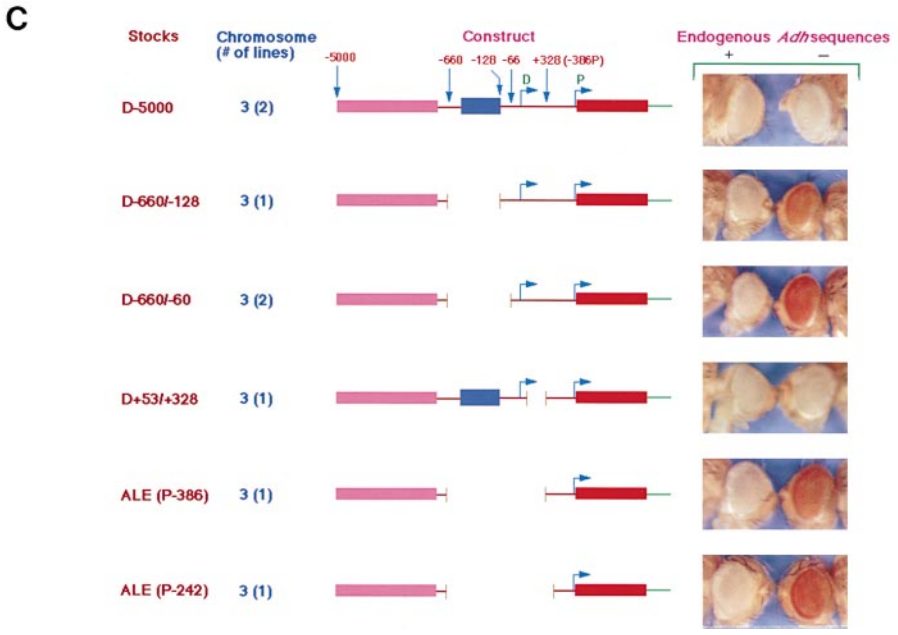


Figure 3. Homology Recognition Sequences that Promote Silencing

(A) The eye color of *Adh-w* flies is reduced in the presence of two copies of full-length *Adh* transgenes. The copy number of transgenes is in parentheses. Note that in this case, deletion of endogenous sequences has no influence on cosuppression.

(B) A Northern blot hybridization using the same genotypes (shown in Figure 3A) as a source of total cellular RNA. The blot was probed with *white* antisense RNA and then reprobed with β-*tubulin* as a loading control. The mean of triplicate ratios of each lane is presented by the bar diagram.

(C) The structure of a series of truncated *Adh* promoter constructs (not to scale) and their effect on *Adh-w* eye color in the presence or absence of *Adh* endogenous sequences. Deletion endpoints are numbered relative to the transcriptional start point of the distal promoter. The larval (red box) and adult enhancer (blue box), promoters (D, distal; P, proximal), the chromosomal sites of each construct, and the number of tested transformant lines are indicated. The deletion of the *Adh* gene restores normal eye pigmentation with four of the truncated constructs. One deleted construct, D+53/+328, produces equal levels of eye pigmentation in both circumstances in a similar manner to the full-length *Adh* transgene.

minimum regulatory sequences for homology recognition between *Adh-w* and *Adh* transgenes. We combined the available constructs with *Adh-w* in a background deleted for the entire *Adh* endogenous sequences to ensure a direct communication via the *Adh* regulatory regions. The eye color of *Adh-w* flies, as well as the *white* transcript levels, was examined in the presence of each construct. Four of the five constructs fail to cosuppress *Adh-w* expression (Figures 3C and 4A). An overlapping deletion map of the truncated regions suggests that a minimum of 532 bp (from −660 to −128 bp before the distal transcriptional start site) is required

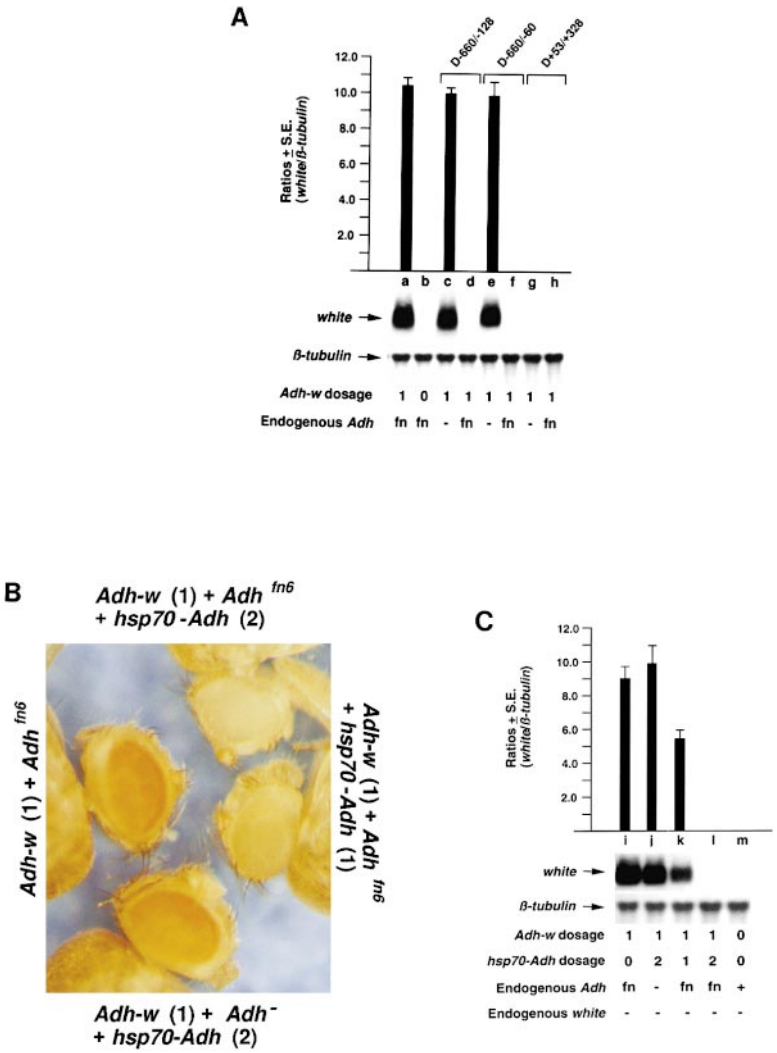


Figure 4. Sequence Homology versus Transgene Expression Requirement for Silencing

(A) A Northern blot hybridization showing that addition of each truncated *Adh* promoter construct in the *Adh-w*/+ flies causes altered levels of *Adh-w* expression depending on the presence or absence of the endogenous *Adh* sequences. The construct used in each lane is noted above. The relative amount of the *Adh* transcripts (*Adh*/β-*tubulin*) from triplicate blots is presented by the bar diagram.

(B) The eye color of *Adh-w* flies is reduced by introducing two copies of *hsp70-Adh* in the presence of endogenous *Adh*. Deletion of the endogenous sequences restores the eye color. The copy number of transgenes is in parentheses. Note that the distal enhancer sequence is required for homology recognition rather than expression of *Adh* RNA from a silencing transgene.

(C) Northern analysis of the same genotypes (as in Figure 4B) showing a similar effect of *hsp70-Adh* transgene on transcript levels. The relative amount of the *Adh* transcripts (*Adh*/β-*tubulin*) from triplicate blots is represented by the bar diagram above the panel.

(Figure 3C). The minimum sequence (532 bp) for homology recognition includes the adult enhancer region of the *Adh* locus.

Although certain truncated *Adh* constructs cannot cause silencing alone, they can all condition cosuppression in the presence of endogenous *Adh* sequences. Thus, the structural gene portion of these transgenes triggers the response, using the endogenous *Adh* as an intermediary. This result demonstrates that the failure of each type of transgene to suppress in the absence of the endogenous *Adh* is unlikely to be due to a position effect.

Homology Recognition and the Adult Enhancer

To determine whether the adult enhancer sequence per se is required for the *w-Adh/Adh-w* interaction or whether diminution of *Adh* expression due to enhancer deletion eliminates the silencing, we used an *hsp70-Adh* fusion construct that carries a constitutive promoter but lacks adult *Adh* enhancer sequences (Corbin and Maniatis, 1989b). Introduction of a single *hsp70-Adh* to *Adh-w*/+ flies in either of two locations (16B and 70C) reduces the eye pigment as well as *white* transcripts to approximately 50% of the normal levels (Figures 4B and 4C). Two copies of the same *hsp70-Adh* construct sharply reduces the eye pigment and *white* transcripts to background levels. The reduction is quite similar to the *Adh-w* silencing produced by multiple *w-Adh* transgenes.

We next generated a genotype that contains two copies of *hsp70-Adh* and a copy of *Adh-w#1* together with the deletions for the *Adh* sequences (Figures 4B and 4C). Similar to the results with the *w-Adh* construct, the constitutively expressed *hsp70-Adh* does not silence the *Adh-w* gene in the *Adh* deletion background. This result indicates that gene expression per se of the endogenous *Adh* or *Adh* transgenes is not the critical factor for the *Adh-w/w-Adh* interaction, but rather, that the specific DNA sequence of the adult enhancer is required to be present.

Developmental Initiation of Nonhomologous Cosuppression

We examined *white* mRNA levels using in situ hybridization of developing embryos to determine the time of onset for *Adh-w* silencing. In *Adh-w* embryos, *white* transcripts were initially detected at the blastoderm stage, about 2.5 hr after egg laying (AEL). The residual

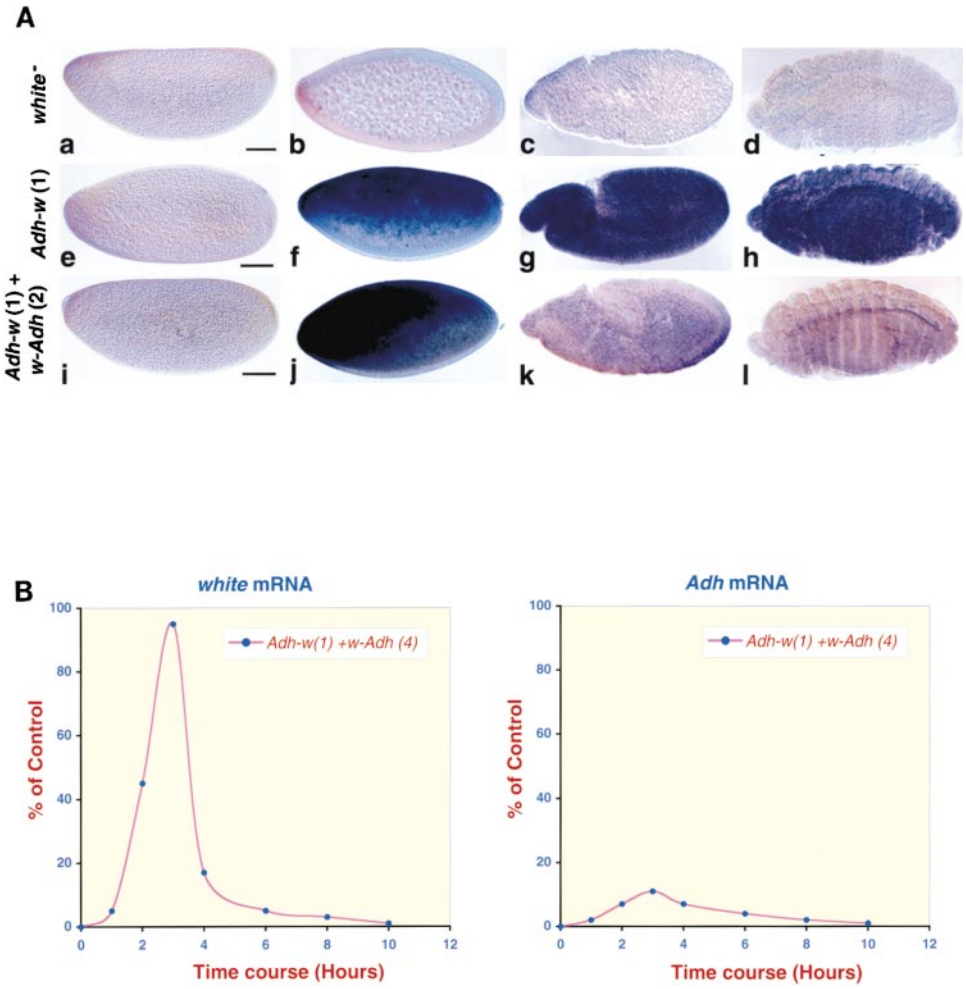


Figure 5. Initiation of Cosuppression during Embryogenesis

(A) The intensity of the purple staining represents the amount of *white* transcripts. (a) Early stage (1 hr after egg laying [AEL]) in *white*$^{-}$ embryos; (b) syncytial blastoderm (2 hr AEL); (c) during germband formation (4 hr AEL); (d) germband retraction (9.5 hr AEL); (e–h) same stages of embryos with a single *Adh-w#1* showing strong expression throughout development starting from the blastoderm stage; (i–l) similar stages for *Adh#1*; *w-Adh#3/w-Adh#3* embryos showing initiation of cosuppression as illustrated by reduction in staining intensity. Bar, 50 μm.

(B) Summary of the initiation of nonhomologous cosuppression in the *Adh-w#1*; *w-Adh* (4) genotype. The probes are noted in each panel. Percentage of embryos are noted in which staining is the same as one copy of *Adh-w* for the *w* probe or one copy of *w-Adh* for the *Adh* probe. The results indicate that the *Adh-w* construct in this genotype achieves briefly an expression similar to an *Adh-w* copy alone, while the four *w-Adh* copies are silenced from the beginning of development relative to a single *w-Adh*.

stain of the w^{-} genotype represents the background (Figure $5A_{a–d}$). We compared the *white* transcript levels of the *Adh-w* alone and cosuppressed embryos that also have two copies of *w-Adh*. In the *Adh-w#1* embryos (Figure $5A_{e–h}$), the anterior end of the ventral furrow, the posterior midgut plate, and the anterior part of the elongating germband are prominently stained during early gastrulation. Later-staged embryos showed a similar level of staining. In the *Adh-w* embryos that carry two copies of *w-Adh* (Figure $5A_{i–l}$), the *white* transcripts are present at a high level at the blastoderm stage but decline to only a trace at gastrulation. A clear reduction of the *white* mRNA was found during germband elongation, which starts at about 3.5 to 4 hr AEL. The quantity of *white* mRNA is basically at the null level by the time of dorsal closure (about 13 hr AEL). Because there is no staining in the earliest embryonic stages, the RNA detected from *Adh-w* in the various genotypes must represent zygotic gene activity rather than a maternal RNA contribution.

We also tested the embryonic expression pattern of *Adh* transcripts produced by various genotypes using Adh^{fn6} as the background control. In *w-Adh*/+ embryos, *Adh* was uniformly expressed at a high level at the blastoderm stage; this level persists throughout embryogenesis (not shown). To determine the initial stage of *w-Adh* cosuppression, we compared the *Adh* transcripts of a single *w-Adh* transgene with that of four copies of *w-Adh* alone and in the *Adh-w#1*; *w-Adh(4)* genotype. In the latter two, the *Adh* transcripts are very low from the beginning of development (Figure 5B). These results suggest that *w-Adh* cosuppression is initiated at the earliest synthesis stage, while *Adh-w* becomes silenced subsequently.

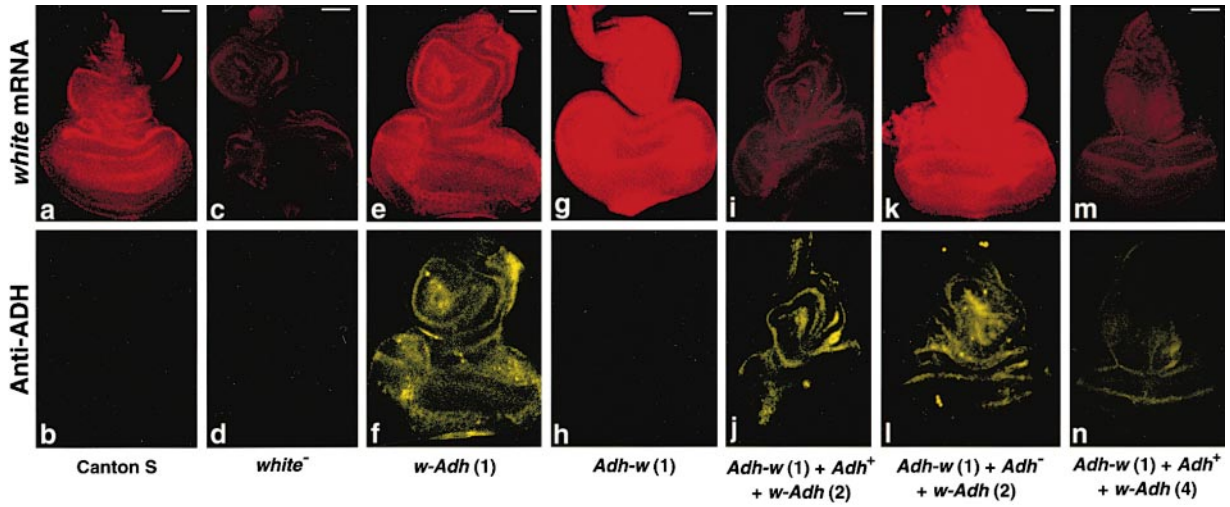


Figure 6. Nonhomologous Cosuppression in a Single Tissue

Nonhomologous cosuppression in the eye antennal imaginal disc. Using FISH, the eye antennal discs were double probed with *white* mRNA (red) and anti-ADH (yellow). (a and b) Canton S; (c and d) *y* w^{67c23}; (e and f) Adh^{fn6}/Adh^{fn6}; *w-Adh#3*/+; (g and h) *y* w^{67c23} *Adh-w#1*/Y; Adh^{fn6}/Adh^{fn6}; (i and j) *Adh-w#1*/Y; Adh^{fn6}/Adh^{fn6}; *w-Adh#3/w-Adh#3*; (k and l) *Adh-w#1*/Y; $Df(2L)Adh^{fn3}/Df(2L)Adh^{379}$; *w-Adh#3/w-Adh#3*; (m and n) *Adh-w#1*/Y; *w-Adh#2/w-Adh#2*; *w-Adh#3/w-Adh#3* larvae. No trace of ADH is detected from the endogenous gene in Canton S and w^{-}. Under silencing conditions (i, j and m, n), *white* RNA is strongly reduced and ADH labeling is characteristic of the cosuppressed level of *w-Adh* copies. Bar, 100 μm.

Nonhomologous Cosuppression in a Single Tissue

With *w-Adh/Adh* cosuppression, silencing occurs in all tissues, as revealed by the histochemical staining of larvae (Pal Bhadra et al., 1997). The results described above revealed that *w-Adh* silencing precedes that of nonhomologous cosuppression. In order to determine further the relationship between the two, we used fluorescence in situ hybridization (FISH) to visualize the expression of *white* RNA and ADH protein in eye antennal discs, using *white* antisense RNA and anti-ADH antibody probes. The expression of each product was assayed in different transgenic stocks and compared to the wild-type levels. In Canton S wild type, *white* is strongly expressed in the eye antennal disc, but no trace of ADH was detected (Figures 6a and 6b). The ADH protein is present at a high level in the gut tissues of the same individuals (not shown). A *white* deficiency stock provides the background control (Figures 6c and 6d). In contrast, ADH proteins are deposited in the eye antennal disc together with *white* mRNA in the single-copy *w-Adh* transformants (Figures 6e and 6f). In the single-copy *Adh-w* transformants, *white* is transcribed at a high level in the disc cells (Figures 6g and 6h). This result suggests that the *w* sequences present contribute to the tissue-specific expression of this construct. We also examined the distribution of the *white* and ADH products in the cosuppressed larvae that carry one copy of the *Adh-w* transgene with two copies of *w-Adh*. As predicted from the larval Northern analysis, the *white* transcripts are reduced to the *white* null levels (Figure 6i). However, the ADH expression is at the level characteristic of two copies of *w-Adh* (Figure 6j), that is, cosuppressed in that it is equivalent to or less than one dose of the construct (Pal Bhadra et al., 1997). We also examined similar transformant larvae that remove the entire *Adh* sequences using the overlapping *Adh* deficiencies. An examination of the eye antennal disc shows that ADH is distributed with an intensity equal to that of cosuppressed stocks, while *white* expression is restored to the normal *Adh-w* level (Figures 6k and 6l). Increasing the copy number of *w-Adh* lowers the total expression of ADH (Figure 6n). Therefore, nonhomologous cosuppression is present in a tissue that lacks endogenous *Adh* mRNA, suggesting that expression of the endogenous *Adh* gene is not required for nonhomologous cosuppression under these circumstances. Morever, cosuppression of multiple *w-Adh* copies is independent of nonhomologous cosuppression. As noted above, nonhomologous cosuppression can be independent of *w-Adh* silencing because it can be initiated by a single active copy of *w-Adh* present in the genome.

Polycomb Association and Nonhomologous Cosuppression

Because PcG proteins are involved with cosuppression of *w-Adh* (Pal Bhadra et al., 1997), we examined the association of the Pc protein with the *Adh* and *Adh-w* insertion sites in the normal and deleted *Adh* flies under cosuppressing conditions. The cytological location of each insert (*Adh-w#1* at 16B region and *Adh-w#2* in 70C region) was determined by in situ hybridization (Schmidt et al., 1988). Immunolocalization of the Pc protein in the polytene chromosomes from strains with single inserts (*Adh-w#1* or *Adh-w#2*) showed that Pc protein binds to more than 100 sites in the polytene chromosomes, as previously noted (Frank et al., 1992; Rastelli et al., 1993), but was not detected at either *Adh-w* site (Figures 7a and 7d). However, labeling under cosuppressing conditions (*y* w^{-} *Adh-w#1/Y*; Adh^{fn6}/Adh^{fn6}; *w-Adh#3/w-Adh#3* or *y* w^{-}; Adh^{fn6}/Adh^{fn6}; *w-Adh#3/Adh-w#2*) revealed that Pc protein is strongly recruited to each *Adh-w* insert (Figures 7b and 7e). No other alteration in Pc banding

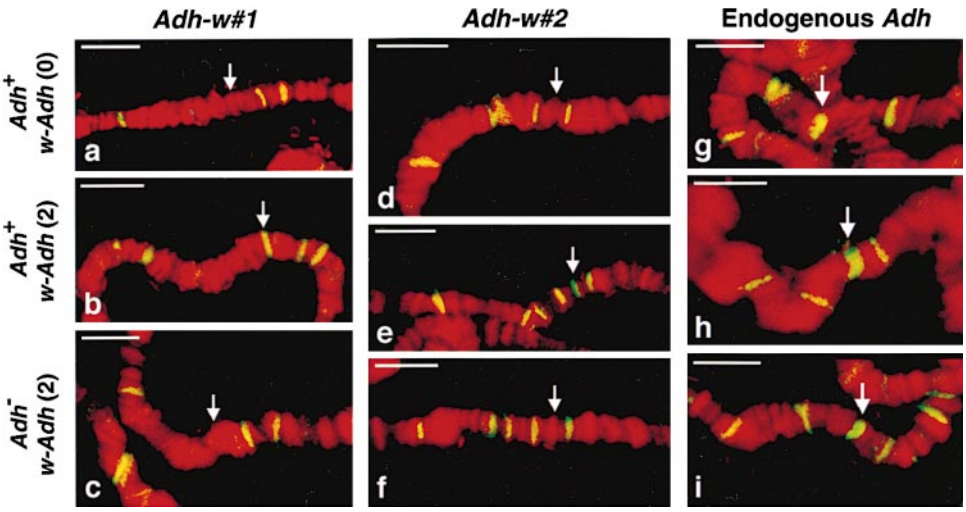


Figure 7. Silenced Transgenes Are Associated with the Polycomb Complex

The cytological locations of the *Adh-w* inserts were determined by in situ hybridization. *Adh-w#1* is at 16B and *Adh-w#2* at 70C. The localization of Pc protein on the *Adh* and *Adh-w* sites was determined by immunostaining of polytene chromosomes followed by confocal microscopy. The chromosomes were stained with propidium iodide (red) and Pc antibodies with Cy-5 (green; not in figure). The yellow regions of the superimposed images indicates association of Pc protein. Arrows indicate the location of an *Adh-w* construct (a–f) or the site of the endogenous *Adh* (g–i). (a) Immunostained X chromosomal segment of the 16B region from an *Adh-w#1*/Y larva; (b) the confocal image of the same region from an *Adh-w#1*/Y larva carrying *w-Adh#3*/*w-Adh#3*; (c) same region of an X chromosome of an *Adh-w#1*/Y; *w-Adh#3*/*w-Adh#3* larva in which the endogenous *Adh* sequence is completely deleted; (d) the 3L chromosomal segment, including the 70C region from an *Adh-w#2*/+ larva; (e) same 3L region from an *Adh-w#2*/+ larva carrying one copy of the *w-Adh#3* insert; (f) 3L chromosomal region of an *Adh-w#2*/+ larva in which the endogenous *Adh* gene is deleted but carrying one copy of *w-Adh#3*; (g) 2R chromosomal segment of the same genotype as (a), including the endogenous *Adh* region (35D); (h) 2R chromosome from the same genotype as (b); and (i) 2R chromosomal segment of the same genotype as (c), showing that deletion of *Adh* sequences does not disrupt the association of Pc protein at 35D. Bar, 10 μm.

pattern was found in these genotypes. We also examined the Pc binding in the polytene chromosomes of the two *Adh-w* stocks in which endogenous *Adh* sequences are deleted but that also carried one or two copies of *w-Adh* (*y* w^- *Adh-w#1*/Y; $Df(2L)Adh^{379}/Df(2L)Adh^{fn3}$; *w-Adh#3*/*w-Adh#3*; and *y* w^-/Y; $Df(2L)Adh^{379}/Df(2L)Adh^{fn3}$; *Adh-w#2*/*w-Adh#3*). Under these circumstances, the Pc proteins failed to associate with the *Adh-w* sites (Figures 7c and 7f) but retained their association near the endogenous *Adh* (Figures 7g–7i). The deletion of the entire *Adh* sequence does not eliminate Pc binding at 35D. Thus, the major site for normal Pc binding in this region is not in *Adh* itself but is sufficiently close to obscure a cytological determination of any changes at *Adh*.

Discussion

In this report, we describe a case of cosuppression between nonhomologous transgenes. Our results suggest that any two unrelated transgenes that share homology with different but contiguous sequences within the genome might be susceptible to silencing. Nevertheless, our interpretation of the interaction does indeed involve homologous recognition with the endogenous *Adh* gene acting as the intermediary. However, homology alone cannot be the only trigger, because the endogenous *white* gene cannot support the interaction. The involvement of the PcG complex suggests a mechanism by which the regions of homology between *w-Adh* and the distal part of the endogenous *Adh* gene are recognized to initiate silencing. Polycomb silencing can spread along the chromatin fiber from the major sites of association (Polycomb response elements [PREs]) to silence multiple genes in *cis* (Paro, 1990; Fauvarque and Dura, 1993; McCall and Bender, 1996; Paro and Harte, 1996; Mallin et al., 1998). Also, Polycomb silencing has recently been shown to operate in *trans* between related transgenes (Hagstrom et al., 1997; Sigrist and Pirrotta, 1997). Thus, one potential mechanism is that the Polycomb complex is first established in the distal *Adh* regions in common between *w-Adh* and *Adh* and then spreads from the distal to the proximal region of the endogenous *Adh* gene. The complex then becomes associated with the reciprocal construct in *trans* to produce the observed silencing. The results suggest that Polycomb silencing is initiated by the presence of *w-Adh* inserts.

These results raise interesting questions about the nature of PREs. These DNA sequences accumulate Pc complex at numerous sites in the chromosomes normally or when transformed back into flies (Chan et al., 1994). Our data indicate that binding of high levels of Pc complex can be initiated and maintained at ectopic positions. There is no obvious PRE sequence, as defined by a consensus (Mihaly et al., 1998) or by the *pleiohomeotic*-binding site (Brown et al., 1998), present in the region of the *Adh* promoter that is required for the interaction with *Adh-w*.

One could perhaps argue that weak PREs exist in *Adh*. If this is the case, then they must exist in both parts of the *Adh* gene. However, even on this hypothesis the tendency for a high level of binding must spread in the endogenous *Adh* gene, because the single *Adh-w* stock without *w-Adh* has the same genetic configuration

at the endogenous *Adh* locus but does not exhibit detectable Pc binding or silencing. Only when *w-Adh* is present in the genome together with the endogenous *Adh* is silencing and high levels of Pc association observed on *Adh-w*. Therefore, the results suggest Pc complex association can spread in *cis* and then in *trans* to previously naive sites based on the underlying DNA homology (or chromatin array dictated by DNA).

The direct interaction between full-length *Adh* constructs and *Adh-w* in the absence of the endogenous *Adh* allowed us to determine those sequences that are required to incite silencing. The identified region lies outside the transcribed portion of *Adh* and surrounds the distal enhancer. Replacement of this region with the constitutively active *hsp-70* promoter does not restore silencing, illustrating the requirement of the sequence per se for silencing rather than the need for expression of the *Adh* coding sequences. Because this region of *Adh* is not known to be transcribed, an RNA-mediated mechanism appears to be unlikely. Moreover, it is doubtful that any ectopic transcription of this region is responsible for silencing, because all insertions in the genome that carry this sequence are effective and the *Adh* gene is present in the opposite transcriptional orientation to the *rosy* eye color transformation marker gene carried on the same transposons (Corbin and Maniatis, 1989b). Also, the pairing configuration of *w-Adh* affects the magnitude of its own silencing, as previously shown (Pal Bhadra et al., 1997), which would not be predicted by an RNA mechanism. Thus, a DNA homology recognition mechanism is likely the basis of the silencing interaction.

An RNA involvement would require action in the nucleus, which is conceivable (for example, see Voinnet et al., 1998), to trigger PcG accumulation, but the transfer of the silencing signal from the nontranscribed region of *Adh* to *Adh-w* would still seem to require homologous association. Indeed, the silencing is initiated during the blastoderm stage, when homologous chromosomes first begin to pair in *Drosophila* (Hiraoka et al., 1993). Whether a homology recognition associated with this process is involved in silencing would be an intriguing future investigation. Certainly, a transcriptional mechanism is implicated based on the property of the Pc complex to produce chromatin inaccessibility (McCall and Bender, 1996).

Given the effectiveness of double-stranded (ds) RNA for silencing of homologous sequences in *Caenorhabditis elegans* (Fire et al., 1998), *Drosophila* (Kennerdell and Carthew, 1998), and *Paramecium* (Ruiz et al., 1998) and the implication of abnormal (Sijen et al., 1996; English et al., 1997) or dsRNA (Metzlaff et al., 1997; Jorgensen et al., 1998; Waterhouse et al., 1998) in post-transcriptional silencing in plants, it is clear that a mechanism of specific RNA turnover operates in many if not most eukaryotic organisms. Conversely, cases of RIPing in *Neurospora* (Selker, 1999), certain examples of dispersed gene silencing in plants (Matzke and Matzke, 1995; Vaucheret et al., 1998), silencing transfer between alleles in *Ascobolus* (Colot et al., 1996), and pairing-sensitive silencing in *Drosophila* (Kassis et al., 1991; Csink and Henikoff, 1996; Brown et al., 1998) are best interpreted as involving homology recognition at the DNA level. The case of cosuppression in *Drosophila* described here falls into this category and illustrates the presence of multiple mechanisms of dispersed gene silencing in animal species as is the case in other taxa.

Experimental Procedures

Drosophila Strains and Genetic Crosses

We selected *w-Adh* and *Adh-w* transgenic strains for this study based on their minimal positional effects. All stocks not specifically referenced in the text are described in Flybase (http://flybase.bio.indiana.edu). To generate double inserts for *w-Adh* together with *Adh-w*, we used multiple balancer stocks. The *Adh-w#1/Adh-w#1* females were crossed with *Basc*/Y; +/*SM6a*; +/*TM3*, *Ser* males. The F1 females (*Adh-w#1/Basc*; +/*SM6a*; +/*TM3*, *Ser*) were mated to *Basc*/Y; Adh^{fn6}/*In(2LR)Gla*; *w-Adh#3/MKRS*, which is the product of a cross between Adh^{fn6}/Adh^{fn6}; *w-Adh#3/w-Adh#3* females and *Basc*/Y; +/*In(2LR)Gla*; +/*MKRS* males. We selected the F2 males (*Adh-w#1*/Y; Adh^{fn6}/*SM6a*; *w-Adh/TM3*, *Ser*) and females (*Adh-w#1/Basc*; Adh^{fn6}/*SM6a*; *w-Adh/TM3*, *Ser*) that carry both reciprocal constructs. A cross between these males and females produced the desired genotype (*Adh-w#1*; Adh^{fn6}/Adh^{fn6}; *w-Adh#3/w-Adh#3*).

To delete the entire endogenous *Adh* sequences, we used two overlapping deficiency stocks, $Df(2L)Adh^{379}$ and $Df(2L)Adh^{fn3}$, that together result in viable flies. We initially combined *Adh-w#1* with $Df(2L)Adh^{379}$ and *w-Adh#3* with $Df(2L)Adh^{fn3}$. The females of the first combined stock, *Adh-w#1/y* w^-; $Df(2L)Adh^{379}$/*In(2LR)Gla*; *TM3*, *Ser/MKRS*, were crossed to males from the second stock, *y* w^-/Y; $Df(2L)Adh^{fn3}$/*SM6a*; *w-Adh#3/w-Adh#3*. The progeny males and females that carry both the reciprocal fusion constructs (*Adh-w#1* and *w-Adh#3*) were mated together. This cross generates the *Adh-w#1*; $Df(2L)Adh^{fn3}$/$Df(2L)A^{379}$; *w-Adh#3/w-Adh#3* stock.

Northern Analysis

RNA isolation from whole flies, gel electrophoresis, and hybridization with antisense and sense RNA probes was as described (Hiebert and Birchler, 1994). Blots were analyzed in triplicate and were reprobed with antisense β*1-tubulin* RNA as a gel loading control. Quantification was performed using a Fuji 2000 Bas phosphorimager.

Histology: In Situ Hybridization

Denatured digoxigenin-labeled probes were generated by random priming and in vitro transcription procedures. All subsequent steps of detection were performed using the Genius Kit (Boehringer Mannheim, Indianapolis, IN). The hybridization and posthybridization washes were carried out as described (Schmidt et al., 1988).

Embryos were collected, dechorionated, fixed, and processed for in situ hybridization as described by Tautz and Pfeifle (1989). For double labeling, the templates for *w* cDNA and the DP fragment of *Adh* (Pal Bhadra et al., 1997) were labeled with digoxigenin-dUTP (Boehringer Mannheim) and biotin-dCTP (GIBCO-BRL) and detected by alkaline phosphatase.

For in situ hybridization, eye antennal disc and larval tissues were dissected and fixed in 4% formaldehyde, 0.1% Tween-20, and 0.1% sodium deoxycholate in PBS for 30 min. Subsequently, tissues were processed as described (Tautz and Pfeifle, 1989). Double detection in the disc and gut tissue was performed using digoxigenin-labeled antisense *w* mRNA and anti-ADH antibodies (Meller et al., 1997). For fluorescence color detection, we used antidigoxigenin rhodamine conjugate for *white* mRNA and goat anti-rabbit Cy-5 conjugate for ADH antibodies at a 1:200 dilution. Photographs were taken with a BioRad-600 confocal microscope and Olympus fluorescence AX-3 microscope. The merged photographs were generated using Adobe photoshop 5.0 program.

PCR Analysis

To define the limits of the *Adh* deficiencies molecularly and to confirm the complete removal of *Adh* in the combination, we performed quantitative PCR using genomic DNA from Canton S, $Df(2L)Adh^{379}$/+, $Df(2L)Adh^{fn3}$/+, and $Df(2L)Adh^{379}$/$Df(2L)Adh^{fn3}$. We selected two sets of *Adh* primers (5′-AATTCAAGCTGTCACAAGTAGTGC, 5′-GGG CCAGTTTCTAAAGGTGTTAT and 5′-GCTTTCCAACTTTTCTAGAT TGAT, 5′-CGTATTTATAGGAAGATCCGTACA) for this study. The first set amplifies 1200 bp, from −551 to +649, while the second

set amplifies 1360 bp, from −1453 to −93 from the transcriptional start site (+1). A pair of primers (5′-GATCTTTAGTTCGGATAGGGT GAC, 5′-TGCTTAAAAGCAACGATATACTGG) was used in each reaction to amplify a 600 bp sequence from +1489 to +2089 from the transcriptional start site of the *rosy* eye color gene as an internal control. In Canton S, both fragments from *Adh* and the *ry* gene are equally amplified, while in the double deficiency stock, only *ry* is amplified. In each single heterozygous deficiency stock, the *Adh* fragment is amplified to approximately 50% of the Canton S level.

Immunostaining of Chromosomes

Immunostaining of the polytene chromosomes with affinity-purified rabbit anti-Pc antibodies was performed as described (Pal Bhadra et al., 1997). Cy-5-conjugated goat anti-rabbit secondary antibody was used. Preparations were mounted with a mixture of Vectashield mounting media and propidium iodide and examined with a Bio-Rad 600 confocal microscope using a 100× oil lens.

Acknowledgments

We thank M. Ashburner, J. Roote, V. Pirrotta, C. Laurie, V. Corbin, and R. Paro for *Drosophila* stocks and antibodies. This work was supported by the National Science Foundation and University of Missouri Research Board grants.

Received June 24, 1999; revised August 20, 1999.

References

Bahramian, M.B., and Zarbl, H. (1999). Transcriptional and posttranscriptional silencing of rodent alpha1(I) collagen by a homologous transcriptionally self-silenced transgene. Mol. Cell. Biol. *19*, 274–283.

Baulcombe, D.C., and English, J.J. (1996). Ectopic pairing of homologous DNA and post transcriptional gene silencing in transgenic plants. Curr. Opin. Biotechnol. *7*, 173–180.

Benyajati, C., Place, A.R., Wang, N., Pentz, E., and Sofer, W. (1982). Deletions at intervening sequence splice sites in the *Alcohol dehydrogenase* gene of Drosophila. Nucleic Acids Res. *10*, 7261–7272.

Birchler, J.A., Hiebert, J.C., and Paigen, K. (1990). Analysis of autosomal dosage compensation involving the *Alcohol dehydrogenase* locus in *Drosophila melanogaster*. Genetics *124*, 677–686.

Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.L., and Kassis, J.A. (1998). The Drosophila Polycomb group gene *pleiohomeotic* encodes a DNA binding protein with homology to the transcription factor YY1. Mol. Cell *1*, 1057–1064.

Chan, C.S., Rastelli, L., and Pirrotta, V. (1994). A Polycomb response element in the *Ubx* gene that determines an epigenetically inherited state of repression. EMBO J. *13*, 2553–2564.

Chaboissier, M.C., Bucheton, A., and Finnegan, D.J. (1998). Copy number control of a transposable element, the I factor, a LINE-like element in Drosophila. Proc. Natl. Acad. Sci. USA *95*, 11781–11785.

Cogoni, C., and Macino, G. (1997). Isolation of quelling-defective (*qde*) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA *94*, 10233–10238.

Cogoni, C., Irelan, J.T., Schumacher, M., Schmidhauser, T.J., Selker, E.U., and Macino, G. (1996). Transgene silencing of the *al-1* gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO J. *15*, 3153–3163.

Colot, V., Maloisel, L., and Rossignol, J.L. (1996). Interchromosomal transfer of epigenetic states in Ascobolus: transfer of DNA methylation is mechanistically related to homologous recombination. Cell *86*, 855–864.

Corbin, V., and Maniatis, T. (1989a). The role of specific enhancer-promoter interactions in the Drosophila *Adh* promoter switch. Genes Dev. *3*, 2191–2200.

Corbin, V., and Maniatis, T. (1989b). Role of transcriptional interference in the *Drosophila melanogaster Adh* promoter switch. Nature *337*, 279–282.

Csink, A., and Henikoff, S. (1996). Genetic modification of heterochromatic association and nuclear organization in Drosophila. Nature *381*, 529–531.

English, J.J., Davenport, G.F., Elmayan, T., Vaucheret, H., and Baulcombe, D.C. (1997). Requirement of sense transcription for homology-dependent virus resistance and *trans*-inactivation. Plant J. *12*, 597–603.

Flavell, R.B. (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA *91*, 3490–3496.

Fauvarque, M.O., and Dura, J.M. (1993). *polyhomeotic* regulatory sequences induce developmental regulator-dependent variegation and targeted P element insertions in Drosophila. Genes Dev. *7*, 1508–1520.

Fire, A., Xu, S., Montgomery, M.K., Kotas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806–811.

Frank, A., Decamillis, M., Zink, D., Cheng, N., Brock, H.G., and Paro, R. (1992). *Polycomb* and *polyhomeotic* are constituents of a multimeric protein complex in chromatin of *Drosophila melanogaster*. EMBO J. *11*, 2941–2950.

Gubb, D., Roote, J., Trenear, J., Coulson, D., and Ashburner, M. (1997). Topological constraints on transvection between *white* genes within the transposing element TE35B in *Drosophila melanogaster*. Genetics *146*, 919–937.

Hagstrom, K., Muller, M., and Schedl, P.A. (1997). *Polycomb* and GAGA dependent silencer adjoins the Fab-7 boundary in the Drosophila *bithorax* complex. Genetics *146*, 1365–1380.

Hiebert, J.C., and Birchler, J.A. (1994). Effects of the *maleless* mutation on X and autosomal gene expression in *Drosophila melanogaster*. Genetics *136*, 913–926.

Hiraoka, Y., Dernburg, A.F., Parmelee, S.J., Rykowski, M.C., Agard, D.A., and Sedat, J.W. (1993). The onset of homologous chromosome pairing during *Drosophila melanogaster* embryogenesis. J. Cell Biol. *120*, 591–600.

Jensen, S., Gassama, M.P., and Heidmann, T. (1999). Taming of transposable elements by homology-dependent gene silencing. Nat. Genet. *21*, 209–212.

Jorgensen, R.A. (1995). Cosuppression, flower color patterns, and metastable gene expression states. Science *268*, 686–691.

Jorgensen, R.A., Atkinson, R.G., Forster, R.L.S., and Lucas, W.J. (1998). An RNA based information superhighway in plants. Science *279*, 1486–1487.

Kassis, J.A., VanSickle, E.P., and Sensabaugh, S.M. (1991). A fragment of *engrailed* regulatory DNA can mediate transvection of the *white* gene in Drosophila. Genetics *128*, 751–761.

Kennerdell, J.R., and Carthew, R.W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the *wingless* pathway. Cell *95*, 1017–1026.

Laurie-Ahlberg, C.C., and Stam, L.F. (1987). Use of P-element mediated transformation to identify the molecular basis of naturally occurring variants affecting *Adh* expression in *Drosophila melanogaster*. Genetics *115*, 129–140.

Mallin, D.R., Myung, J.S., Patton, J.S., and Geyer, P.K. (1998). Polycomb group repression is blocked by the Drosophila *suppressor of Hairy-wing* [*su(Hw)*] insulator. Genetics *148*, 331–339.

Matzke, M.A., and Matzke, A.J.M. (1995). How and why do plants inactivate homologous (trans) genes? Plant Physiol. *107*, 679–685.

Matzke, M.A., Primig, M., Trnovsky, J., and Matzke, A.J.M. (1989). Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J. *8*, 643–649.

McCall, K., and Bender, W. (1996). Probes for chromatin accessibility in the Drosophila *bithorax* complex respond differently to *Polycomb*-mediated repression. EMBO J. *15*, 569–580.

Meller, V.H., Wu, K.H., Roman, G., Kuroda, M.I., and Davis, R.L. (1997). *roX1* RNA paints the X chromosome of male Drosophila and is regulated by the dosage compensation system. Cell *88*, 445–457.

Metzlaff, M., O'Dell M., Cluster, P.D., and Flavell, R.B. (1997). RNA-mediated RNA degradation and *chalcone synthase A* silencing in petunia. Cell *88*, 845–854.

Meyer, P., Heidmann, I., and Niedenhof, I. (1993). Differences in DNA methylation are associated with a paramutation phenomenon in transgenic petunia. Plant J. *4*, 89–100.

Mihaly, J., Mishra, R.K., and Karch, F. (1998). A conserved sequence motif in Polycomb-response elements. Mol. Cell *1*, 1065–1066.

Montgomery, M.K., Xu, S., and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA *95*, 15502–15507.

Napoli, C., Lemieux, C., and Jorgenson, R.A. (1990). Introduction of a chimeric *chalcone synthase* gene in petunia results in reversible cosuppression of homologous genes in trans. Plant Cell *2*, 279–289.

Pal Bhadra, M., Bhadra, U., and Birchler, J.A. (1997). Cosuppression in Drosophila: gene silencing of *Alcohol dehydrogenase* by *white-Adh* transgenes is *Polycomb* dependent. Cell *90*, 479–490.

Paro, R. (1990). Imprinting a determined state into the chromatin of Drosophila Trends Genet. *6*, 416–421.

Paro, R., and Harte, P.J. (1996). The role of *Polycomb* group and *trithorax* group chromatin complexes in the maintenance of determined cell states. In Epigenetic Mechanisms of Gene Regulation, E. Russo, A.D. Riggs, and R.A. Martienssen, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Press) pp. 507–528.

Rabinow, L., Nguyen-Huynh, A.T., and Birchler, J.A. (1991). A trans-acting regulatory gene that inversely affects the expression of the *white*, *brown* and *scarlet* loci in Drosophila. Genetics *129*, 463–480.

Rastelli, L., Chan, C.S., and Pirrotta, V. (1993). Related chromosome binding sites for *zeste*, *suppressors of zeste* and Polycomb Group proteins in Drosophila and their dependence on *Enhancer of zeste* function. EMBO J. *12*, 1513–1522.

Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D., Benz, W.K., and Engels, W.R. (1988). A stable source of P-element transposase in *Drosophila melanogaster*. Genetics *118*, 461–470.

Ruiz, F., Vayssie, L., Klotz, C., Sperling, L., and Madeddu, L. (1998). Homology-dependent gene silencing in Paramecium. Mol. Cell. Biol. *9*, 931–943.

Schmidt, E.R., Keyl, H.G., and Hankeln, T. (1988). *In situ* localization of two haemoglobin gene clusters in the chromosomes of 13 species of Chironomous. Chromosoma *96*, 353–359.

Selker, E.U. (1999). Gene silencing: repeats that count. Cell *97*, 157–160.

Sigrist, C.J.A., and Pirrotta, V. (1997). Chromatin insulator elements block the silencing of a target gene by the Drosophila Polycomb response element (PRE) but allow *trans* interactions between PREs on different chromosomes. Genetics *147*, 209–221.

Sijen, T., Wellink, J., Hiriart, J.B., and van Kammen, A. (1996). RNA mediated virus resistance: role of repeated transgenes and delineation of targeted regions. Plant Cell *8*, 2277–2294.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma *98*, 81–85.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N., and Stuitje, A.R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell *2*, 291–299.

Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C., and Vernhettes, S. (1998). Transgene-induced gene silencing in plants. Plant J. *16*, 651–659.

Voinnet, O., Vain, P., Angell, S., and Baulcombe, D.C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell *95*, 177–187.

Waterhouse, P.M., Graham, M.W., and Wang, M.B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA *95*, 13959–13964.

Zachar, Z., and Bingham, P.M. (1982). Regulation of *white* locus expression: the structure of mutant alleles at the *white* locus of *Drosophila melanogaster*. Cell *30*, 529–541.